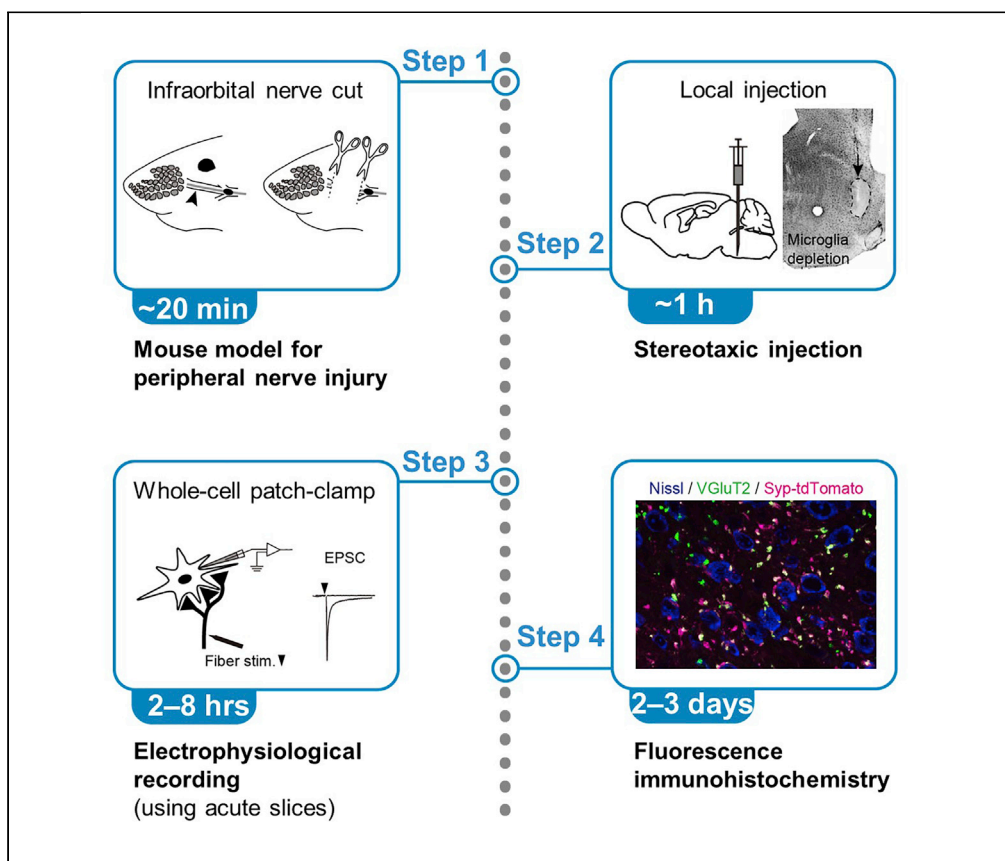**Protocol**

Electrophysiological and anatomical characterization of synaptic remodeling in the mouse whisker thalamus

Yoshifumi Ueta,
Mariko Miyata

yueta@twmu.ac.jp (Y.U.)
mmiyata@twmu.ac.jp
(M.M.)

Highlights

The infraorbital nerve cut for preparing a peripheral nerve injury mouse model

Pressure or iontophoretic drug application via stereotaxic injection

Assessing functional synaptic remodeling by whole-cell patch-clamp in acute slices

Immunohistochemical identification of structural synaptic remodeling

In the central nervous system, developmental and pathophysiologic conditions cause a large-scale reorganization of functional connectivity of neural circuits. Here, by using a mouse model for peripheral sensory nerve injury, we present a protocol for combined electrophysiological and anatomical techniques to identify neural basis of synaptic remodeling in the mouse whisker thalamus. Our protocol provides comprehensive approaches to analyze both structural and functional components of synaptic remodeling.

STAR Protocols

Protocol

Electrophysiological and anatomical characterization of synaptic remodeling in the mouse whisker thalamus

Yoshifumi Ueta[1,2,3,]* and Mariko Miyata[1,]*

[1]Division of Neurophysiology, Department of Physiology, School of Medicine, Tokyo Women's Medical University, Tokyo, 162-8666, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: yueta@twmu.ac.jp (Y.U.), mmiyata@twmu.ac.jp (M.M.)
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SUMMARY

In the central nervous system, developmental and pathophysiologic conditions cause a large-scale reorganization of functional connectivity of neural circuits. Here, by using a mouse model for peripheral sensory nerve injury, we present a protocol for combined electrophysiological and anatomical techniques to identify neural basis of synaptic remodeling in the mouse whisker thalamus. Our protocol provides comprehensive approaches to analyze both structural and functional components of synaptic remodeling.
For complete details on the use and execution of this protocol, please refer to Ueta and Miyata, (2021).

BEFORE YOU BEGIN

Structures and functions of neural circuitry in the central nervous system are dynamically refined during development and in disease. Because neural and glial mechanisms both involve in the induction and maintenance of synaptic remodeling, investigating how neural or glial mechanisms change physiological and morphological properties of synapses and characterizing interactions between neurons and glial cells, such as astrocytes or microglia, are of particular importance to understand neural mechanisms underlying developmental maturation or etiology.

We have recently reported the microglia-dependent mechanism of structural and functional remodeling in the mouse whisker thalamus (Ueta and Miyata, 2021), which is triggered by peripheral sensory nerve injury (Nagumo et al., 2020; Takeuchi et al., 2012; 2017). In that study, we have established region-specific depletion or activation of microglia using stereotaxic injections of microglial inhibitor or activator.

Here, we describe step-by-step procedures for development of mouse model for peripheral nerve injury, stereotaxic injection, and electrophysiological and histological assessment of synaptic remodeling in the whisker thalamus. These approaches include fundamental techniques for mouse surgery, whole-cell patch-clamp using acute brain slices, and immunohistochemistry combined with mouse genetics, and will be useful for studies investigating neural and glial contribution to synaptic remodeling in diverse regions under pathophysiological conditions, and also during development.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-Iba1 antibody (1:500–1,000 dilution)	Abcam	Cat#ab5076; PRID: AB_2224402
Rabbit Polyclonal Anti-Iba1 Antibody (1:500–1,000 dilution)	FUJIFILM Wako Pure Chemical Corporation	Cat#019-19741; PRID: AB_839504

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit polyclonal anti-NeuN antibody (1:1,000–5,000 dilution)	Merck (Sigma-Aldrich)	Cat#ABN78; PRID: AB_10807945
Mouse monoclonal anti-NeuN antibody (1:1,000–2,000 dilution)	Merck (Sigma-Aldrich)	Cat#MAB377; PRID: AB_2298772
Rabbit polyclonal anti-TMEM119 antibody (1:500–1,000 dilution)	Abcam	Cat#ab209064; PRID: AB_2800343
Guinea Pig Polyclonal Anti-VGluT2 Antibody (1:500–1,000 dilution)	Nittobo Medical Co., Ltd. (transferred from Frontier Institute Co., Ltd.)	Cat#MSFR106290; PRID: AB_2341096
Guinea Pig Polyclonal Anti-VGluT2 Antibody (1:1,000–5,000 dilution)	Merck (Sigma-Aldrich)	Cat#AB2251-I; PRID: AB_2665454
Rabbit Polyclonal Anti-VGluT2 Antibody (1:500–1,000 dilution)	Nittobo Medical Co., Ltd. (transferred from Frontier Institute Co., Ltd.)	Cat#MSFR106310; PRID: AB_2619683
Donkey anti-Guinea Pig IgG Secondary Antibody, Alexa Fluor 488 Conjugate (1:500 dilution)	Jackson ImmunoResearch Laboratories	Cat#706-545-148; PRID: AB_2340472
Donkey Anti-Guinea Pig IgG Secondary Antibody, Alexa Fluor 647 Conjugate (1:500 dilution)	Jackson ImmunoResearch Laboratories	Cat#706-605-148; PRID: AB_2340476
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488 conjugate (1:500 dilution)	Thermo Fisher Scientific	Cat#A21206; PRID: AB_2535792
Chemicals, peptides, and recombinant proteins		
Agar	FUJIFILM Wako Pure Chemical Corporation	Cat#010-15815; CAS: 9002-18-0
Agarose S	Nippon Gene Co., Ltd.	Cat#312-01193; CAS: 9012-36-6
Atipamezole (Antisedan)	Nippon Zenyaku Kogyo Co., Ltd.	CAS: 104075-48-1
Bicuculline methochloride	Tocris	Cat#0131; CAS: 53552-05-9
Biocytin	Toronto Research Chemicals Inc. or Sigma	Cat#B388100 or #B4261, CAS: 576-19-2
Butorphanol (Vetorphale)	Meiji Seika Pharma Co., Ltd.	CAS: 42408-82-2
$CaCl_2 \cdot 2H_2O$	Kanto Chemical Co., Inc.	Cat#07058-00; CAS: 10035-04-8
CGP55845	Tocris	Cat#1248; CAS: 149184-22-5
Cholera toxin subunit B, Alexa Fluor 555 conjugate	Thermo Fisher Scientific	Cat#C34776
Cholera toxin subunit B, Alexa Fluor 647 conjugate	Thermo Fisher Scientific	Cat#C34778
Chromium (III) potassium sulfate $\cdot 12H_2O$	Kanto Chemical Co., Inc.	Cat#07360-00; CAS: 7788-99-0
Clodronate liposomes	Katayama Chemical	Cat#160-0430-1
$CsMeSO_3$	Merck (Sigma-Aldrich)	Cat#C1426; CAS: 2550-61-0
CsOH 50 wt. % solution in water	Merck (Sigma-Aldrich)	Cat#232041; CAS: 21351-79-1
D-(+)-glucose	FUJIFILM Wako Pure Chemical Corp.	Cat#049-31165; CAS: 50-99-7
$EDTA \cdot 2Na \cdot 2H_2O$	Nacalai Tesque	Cat#15111-45; CAS: 6381-92-6
EGTA	Nacalai Tesque	Cat#15214-92; CAS: 67-42-5
Ethylene glycol	Merck (Sigma-Aldrich)	Cat#09-1540-5; CAS: 107-21-1
Fluoromount-G (refractive index, ~1.4)	SouthernBiotech	Cat#0100-01
Glycerol	Nacalai Tesque	Cat#17017-35; CAS: 56-81-5
HCl (1 M)	FUJIFILM Wako Pure Chemical Corporation	Cat#083-01095; CAS: 7647-01-0
Heparin sodium salt from porcine intestinal mucosa	Merck (Sigma-Aldrich)	Cat#H3393; CAS: 9041-08-1
HEPES	Merck (Sigma-Aldrich)	Cat#H3375; CAS: 7365-45-9
Isoflurane	Viatris (Mylan N.V.)	CAS: 26675-46-7
KCl	FUJIFILM Wako Pure Chemical Corporation	Cat#163-03545; CAS: 7447-40-7
K-gluconate	Merck (Sigma-Aldrich)	Cat#G4500; CAS: 299-27-4
KOH (1 M)	FUJIFILM Wako Pure Chemical Corporation	Cat#169-03885; CAS: 1310-58-3
Lactic acid	Nacalai Tesque	Cat#20006-62; CAS: 50-21-5
Lipopolysaccharides from E.Coli O26	FUJIFILM Wako Pure Chemical Corporation	Cat#120-05131; CAS: 93572-42-0

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Medetomidine (Domitor)	Nippon Zenyaku Kogyo Co., Ltd.	CAS: 86347-15-1
$MgCl_2 \cdot 6H_2O$	FUJIFILM Wako Pure Chemical Corporation	Cat#135-00165; CAS: 7791-18-6
Midazolam	Sandoz K.K.	CAS: 59467-70-8
myo-Inositol	FUJIFILM Wako Pure Chemical Corporation	Cat#094-00281; CAS: 87-89-8
Na_2-ATP	Merck (Sigma-Aldrich)	Cat#A7699; CAS: 34369-07-8
$Na_2HPO_4 \cdot 12H_2O$	FUJIFILM Wako Pure Chemical Corporation	Cat#196-02835; CAS: 10039-32-4
NaCl	FUJIFILM Wako Pure Chemical Corporation	Cat#191-01665; CAS: 7647-14-5
Na-GTP	Merck (Sigma-Aldrich)	Cat#G8877; CAS: 36051-31-7
$NaH_2PO_4 \cdot 2H_2O$	FUJIFILM Wako Pure Chemical Corporation	Cat#192-02815; CAS: 13472-35-0
$NaHCO_3$	FUJIFILM Wako Pure Chemical Corporation	Cat#191-01305; CAS: 144-55-8
NaOH (1 M)	FUJIFILM Wako Pure Chemical Corporation	Cat#192-02175; CAS: 1310-73-2
Neurobiotin	Vector Laboratories	Cat#SP-1120; CAS: 111822-45-8
NeuroTrace™ 435/455 Blue Fluorescent Nissl Stain (1:100–200 dilution)	Thermo Fisher Scientific	Cat#N21479
Normal donkey serum	ImmunoBioScience Corp.	Cat#IHR-8135
Ofloxacin gel (Tarivid opthalamic ointment 0.3%)	Santen Pharmaceutical Co., Ltd.	CAS: 82419-36-1
Paraformaldehyde	TAAB laboratories Equipment Ltd.	Cat#P001; CAS: 30525-89-4
Picric acid	Nacalai Tesque	Cat#27926-02; CAS: 88-89-1
PLX3397 (Pexidartinib)	Selleck Chemicals	Cat#S7818; CAS: 1029044-16-3
ProLong™ Gold Antifade Mountant (refractive index, 1.47)	Thermo Fisher Scientific	Cat#P36934
QX314 (lidocaine N-ethyl bromide)	Merck (Sigma-Aldrich)	Cat#L5783, CAS: 21306-56-9
Sevoflurane	Viatris (Mylan N.V.)	CAS: 28523-86-6
SlowFade™ Diamond Antifade Mountant (refractive index, 1.47)	Thermo Fisher Scientific	Cat#S36972
Sodium ascorbate ((+)-sodium L-ascorbate)	Merck (Sigma-Aldrich)	Cat#A7631; CAS: 134-03-2
Sodium pyruvate (pyruvic acid sodium salt)	Nacalai Tesque	Cat#29806-12; CAS: 113-24-6
Spongel	LTL Pharma	N/A
Streptavidin, Alexa Fluor 488 conjugate (1:500 dilution)	Thermo Fisher Scientific	Cat#S11223
Sucrose	FUJIFILM Wako Pure Chemical Corporation	Cat#196-00015; CAS: 57-50-1
Thiourea	FUJIFILM Wako Pure Chemical Corporation	Cat#204-01202; CAS: 62-56-6
Tris base (Trizma base)	Merck (Sigma-Aldrich)	Cat#T6066; CAS: 77-86-1
Tris-HCl	Nacalai Tesque	Cat#35433-15; CAS: 1185-53-1
Trisodium citrate dihydrate	FUJIFILM Wako Pure Chemical Corporation	Cat#204-16675; CAS: 6132-04-3
Triton X-100	Nacalai Tesque	Cat#12967-32; CAS: 9002-93-1
Xylocaine jelly 2% (lidocaine hydrochloride)	Aspen Japan K.K.	CAS: 73-78-9
Xylocaine with 1:80,000 adrenaline	Dentsply Sirona K.K.	CAS: 73-78-9 (for lidocaine hydrochloride), 51-43-4 (for adrenaline)
Experimental models: organisms/strains		
Mouse: C57BL/6JJmsSlc	Sankyo Labo Service Corporation Inc.	MGI: 5488963; PRID: MGI:5488963
Mouse: Egr2$^{tm2(cre)Pch}$/J (Krox20-Cre)	The Jackson Laboratory	JAX: 025744; PRID: IMSR_JAX:025744
Mouse: B6;129S-Gt(ROSA) 26Sor$^{tm34.1(CAG-Syp/tdTomato)Hze}$/J (Ai34D)	The Jackson Laboratory	JAX: 012570; RRID: IMSR_JAX:012570
Software and algorithms		
Adobe Photoshop/Illustrator CC	Adobe Inc.	https://www.adobe.com/
Igor Pro 6	WaveMetrics	https://www.wavemetrics.com/
ImageJ	NIH	https://imagej.nih.gov/ij/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NeuroMatic	Rothman and Silver, 2018	http://www.neuromatic.thinkrandom.com/
pClamp 8	Molecular Devices	https://www.moleculardevices.com/
Zen	Carl Zeiss	https://www.zeiss.de/corporate/home.html
Other		
1 or 20 mL syringe	Terumo Corporation	Cat#SS-01T or SS-20ESZ
21-, 26-, or 27-gauge syringe needle	Terumo Corporation	Cat#NN-2138R, NN-2613S, or NN-2719S
35G beveled needle	World Precision Instruments	Cat#NF35BV-2
Anesthetic apparatus with anesthesia induction chamber	Shinano MFG Co. Ltd.	Cat#SN-487-0T
Aron Alpha (cyanoacrylate)	Toagosei Co. Ltd.	Cat#31204
Concentric stimulating electrode	Inter Medical Co. Ltd.	Cat#IMB-160820
Confocal laser scanning microscope	Carl Zeiss	Cat#LSM710
Cooled CCD camera (for histology)	Quantum Scientific Imaging, Inc.	Cat#QSI RS 6.1
Coverslip (24 × 32, 40, 50, and 60 mm)	Matsunami Glass Ind. Ltd.	Cat#C024321, C024401, C024501, and C024601
DC temperature controller (for surgery)	FHC	Cat#40-90-8D
Digitizer	Molecular Devices	Cat#Digidata 1322A
Electrical tape	Yamato Co. Ltd.	Cat#NO200-19
Electronic stimulator	Nihon Kohden	Cat#SEN-7203
Epifluorescence microscope (for electrophysiology)	Olympus Corporation	Cat#BX51WI
Epifluorescence microscope (for histology)	Carl Zeiss	Cat#Axio Scope.A1
Flexible needle	World Precision Instruments	Cat#MF28G-5
Flow tube for peristaltic pump (for electrophysiology; I.D., 1.29 mm)	M&S Instruments Inc.	F117940
Flow tube for peristaltic pump (for paraformaldehyde fixation; I.D., 2.28 mm)	M&S Instruments Inc.	F117946
Fluorescence microscope	Keyence	Cat#BZ-X810
Glass capillary (O.D., 1.0 and I.D., 0.6 mm)	Narishige	Cat#G-1
Glass capillary (O.D., 1.5 and I.D., 0.9 mm)	Narishige	Cat#G-1.5
Glass capillary (O.D., 1.5 and I.D., 0.86 mm)	Sutter Instrument	Cat#B150-86-10
Glass capillary (O.D., 1.5 and I.D., 1.17 mm)	Harvard Apparatus	Cat#30-0062
Glass microscope slides	Matsunami Glass Ind. Ltd.	Cat#S2441
Hamilton syringe (10 μL)	World Precision Instruments	Cat#Nanofil
Heat pad	Natsume Seisakusho Co. Ltd.	Cat#KN-475-2-40
Injector blade (for preparing thin sections of the fixed brain)	Schick Japan K. K.	Cat#SII-10
Iontophoresis pump	Kation Scientific	Cat#BAB-600
Magnetic hot plate stirrer	IKA	Cat#RH B2 S004
Microelectrode amplifier	Molecular Devices LLC.	Cat#Multiclamp 700A
Micromanipulator (for electrophysiology)	Sutter Instrument	Cat#MP-225
Micromanipulator (for stereotaxic injection)	Narishige	Cat#SM-15R/SM-15M-2
Micromotor system	Minitor Co. Ltd.	Cat#Minimo one series ver.2
Micropipette puller	Sutter Instrument	Cat#P-1000 or P-97
Micro-Sample Osmometer	Fiske Associates Inc.	Cat#Model 210
Microsyringe pump controller	World Precision Instruments	Cat#Micro 4
Microtome blade (for trimming the fixed brain)	Feather Safety Razor Co. Ltd.	Cat#A35
Near-infrared camera	Hamamatsu Photonics	Cat#C3077-79
Near-infrared camera controller	Hamamatsu Photonics	Cat#C2741-62
Paraffin oven (laboratory incubator)	Hirasawa Works	Cat#SC-4d-CP
Peristaltic pump	Gilson Inc.	Cat#Minipuls 3
pH indicator paper (pH 5.0–10.0)	Merck	Cat#109533
pH meter	Horiba Ltd.	Cat#B-712
Razor blade (for preparing acute brain slices)	Feather Safety Razor Co. Ltd.	Cat#81-S
Rodent brain matrix	ASI Instruments Inc.	Cat#RBM-2000C
Shaker	TAITEC Corporation	Cat#Mix-VR and NR-80
Steel bur (Midwest carbide bur)	Dentsply Sirona K.K.	Cat#386301

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stereo microscope (for surgery)	Leica Microsystems Inc.	Cat#M60
Stereotaxic apparatus	Narishige	Cat#SR-5M-HT
Stimulus isolator	A.M.P.I.	Cat#Iso-Flex
Surgical blade (for trimming the brain)	Feather Safety Razor Co. Ltd	Cat#21
Syringe filter (0.2 μm, non-sterile)	Millipore (Merck)	Cat#SLLGH04NL
Temperature controller (line heater)	Warner Instruments	Cat#TC-324B
Vibratome	Leica Microsystems Inc.	Cat#VT-1000S and VT-1200S
Vinyl repair patch	Otsuka Corporation	Cat#TGK-280
Water bath system	AS ONE Corporation	Cat#TR1

MATERIALS AND EQUIPMENT

10× artificial cerebrospinal fluid (aCSF) stock solution

Reagent	Final concentration	Amount
NaCl	1.25 M	73.05 g
NaHCO$_3$	260 mM	21.84 g
NaH$_2$PO$_4$·2H$_2$O	12.5 mM	1.95 g
KCl	25 mM	1.86 g
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

The solution can be stored at 4°C for 1 month.

aCSF solution

Reagent	Final concentration	Amount
10× aCSF	1×	100 mL
1 M CaCl$_2$ solution	2 mM	2 mL
1 M MgCl$_2$ solution	1 mM	1 mL
D-(+)-Glucose	20 mM	3.6 g
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

Add 1 M CaCl$_2$ solution and 1 M MgCl$_2$ solution after diluting 10× aCSF solution with ddH$_2$O and bubbled with a mixture of 95% O$_2$ and 5% CO$_2$ for 10–30 min at 20°C–25°C.

Although the solution may be stored at 4°C for one week, we recommend to prepare the fresh solution on the day of use. Osmolarity of the solution is 310 ± 5 mOsm.

pH of the solution falls to 7.2–7.3 after bubbling with a mixture of 95% O$_2$ and 5% CO$_2$ for 10 min at 20°C–25°C .

1 M CaCl$_2$ solution

Reagent	Final concentration	Amount
CaCl$_2$·2H$_2$O	1 M	14.7 g
ddH$_2$O	N/A	Fill up to 100 mL
Total	N/A	100 mL

The solution can be stored at 4°C for at least 3 months.

1 M MgCl$_2$ solution

Reagent	Final concentration	Amount
MgCl$_2$·6H$_2$O	1 M	20.3 g
ddH$_2$O	N/A	Fill up to 100 mL
Total	N/A	100 mL

The solution can be stored at 4°C for at least 3 months.

Cutting solution (sucrose-based solution)

Reagent	Final concentration	Amount
Sucrose	234 mM	80.1 g
$NaHCO_3$	25 mM	2.1 g
$NaH_2PO_4 \cdot 2H_2O$	1.25 mM	0.2 g
KCl	2.5 mM	0.19 g
1 M $CaCl_2$ solution	0.5 mM	0.5 mL
1 M $MgCl_2$ solution	10 mM	10 mL
myo-Inositol	0.5 mM	0.09 g
D-(+)-Glucose	20 mM	3.6 g
ddH_2O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

The composition is originally based on the previous study (Gentet and Ulrich, 2003).
The solution can be stored at 4°C for 1 month.
Osmolarity of the solution is 360 ± 10 mOsm.
pH of the solution falls to 7.1–7.3 after bubbling with a mixture of 95% O_2 and 5% CO_2 for 10 min on ice.

Alternatives: Cold aCSF solution can be used as the cutting solution.

1.25× Cs^+-based pipette internal solution for voltage-clamp recording

Reagent	Final concentration	Amount
$CsMeSO_3$	120 mM	547 mg
NaCl	20 mM	23 mg
QX314	5 mM	34 mg
Na_2-ATP	2 mM	22 mg
Na-GTP	0.5 mM	5 mg
1 M $CaCl_2$ solution	0.1 mM	2 μL
1 M $MgCl_2$ solution	2 mM	40 μL
HEPES	10 mM	48 mg
EGTA	1 mM	8 mg
ddH_2O	N/A	Fill up to 16 mL
Total	N/A	16 mL

Add 15 mL of ddH_2O first and mix.
After pH adjustment to 7.2–7.3 with CsOH, fill up to 16 mL by ddH_2O.
A 0.8-mL aliquot in the 1.5 mL or 2 mL microtube can be stored at −30°C for at least 3 months.
Thaw an aliquot on the day of use, and adjust osmolarity to 290 mOsm by adding ddH_2O.
Filter the solution (nearly 1 mL of total amount) using non-sterile 0.20 μm mini-syringe filter before use.
If necessary, add 0.5% (w/v) biocytin and vortex the solution before adjusting osmolarity. Sonication is effective to prepare the biocytin solution.
Biocytin can also be dissolved in the initial step before aliquot.
Aliquots can be stored at −30°C for at least 3 months as described above.
Once prepared, the solution may be stored at 4°C for a week.

Alternatives: Neurobiotin can be used instead of biocytin for visualizing cell morphology.

1.25× K^+-based pipette internal solution for current-clamp recording

Reagent	Final concentration	Amount
K-gluconate	130 mM	609 mg
Na_2-ATP	2 mM	22 mg
Na-GTP	0.3 mM	3 mg
1 M $MgCl_2$ solution	2 mM	40 μL
HEPES	10 mM	48 mg

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Reagent	Final concentration	Amount
EGTA	0.2 mM	1.6 mg
Biocytin	0.5% (w/v)	100 mg
ddH$_2$O	N/A	Fill up to 16 mL
Total	N/A	16 mL

Prepare the solution similar to the Cs$^+$-based internal solution described above, but adjust pH using KOH.

Biocytin is always included in the K$^+$-based internal solution in our experiments.

A 0.8-mL aliquot in the 1.5 mL or 2 mL microtube can be stored at −30°C for at least 3 months.

Thaw a 0.8-mL aliquot on the day of use, and adjust osmolarity to 290 mOsm by adding ddH$_2$O.

Total amount of the solution becomes nearly 1 mL.

Filter the solution before use as described above.

Once prepared, the solution may be stored at 4°C for a week.

⚠ CRITICAL: As it is difficult to measure the small amount of reagent, you can prepare larger amount of internal solutions or concentrated solution, especially for Na$_2$-ATP, Na-GTP, and EGTA. We adjust osmolarity just before the experiment, because osmolarity may be slightly changed during the storage. For more convenience, you can fill up to 20 mL after pH adjustment and adjust osmolarity to 290 mOsm. In that case, a 1-mL aliquot can be stored, but we recommend to measure osmolarity of thawed solution just before the experiment.

0.1 M phosphate buffer (PB) (pH 7.4)

Reagent	Final concentration	Amount
Na$_2$HPO$_4$·12H$_2$O	80 mM	29 g
NaH$_2$PO$_4$·2H$_2$O	20 mM	2.95 g
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

If pH is lower than 7.4, add few drops of 1 M NaOH and adjust pH to 7.4.

Add 9 g NaCl for preparing phosphate buffered saline (PBS). The solution can be stored at 4°C for 1 year.

0.05 M Tris buffer (TB) (pH 7.6)

Reagent	Final concentration	Amount
Tris-HCl	500 mM	6.06 g
Tris base	11.5 mM	1.39 g
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

Add 0.9 g NaCl for preparing tris buffered saline (TBS). The solution can be stored at 4°C for 1 year.

Prefixative solution (sucrose-based solution)

Reagent	Final concentration	Amount
Sucrose	250 mM	8.5 g
1 M MgCl$_2$ solution	5 mM	0.5 mL
0.1 M PB	20 mM	20 mL
ddH$_2$O	N/A	Fill up to 100 mL
Total	N/A	100 mL

The solution can be stored at 4°C for 1 month.

Alternatives: Saline (0.9% NaCl) can be used as the prefixative solution. To prevent blood coagulation, heparin can be included in the prefixative solution (10–20 units/mL).

Fixative solution (4% paraformaldehyde and 0.2% picric acid)

Reagent	Final concentration	Amount
Paraformaldehyde	4% (w/v)	40 g
Picric acid	0.2% (w/v)	2 g
0.2 M PB	0.1 M	500 mL
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

Add 500 mL 0.2 M PB and 300 mL ddH$_2$O in a 1000-mL conical flask.
Add 40 g paraformaldehyde powder.
Heat the solution at 60°C and stir the solution until paraformaldehyde powder is completely solubilized when the cloudy so-lution becomes clear. It will require 1–2 h. Add 7–9 drops of 1 M NaOH using a 3-mL disposable transfer pipette if necessary.
pH of the solution is adjusted to around 7.5 using a pH indicator paper.
Cool the solution to 4°C by placing the flask on ice.
Add 2 g picric acid powder and stir until the powder is completely solubilized. It will finish within 10 min.
After filtering the solution using a filter paper, fill up to 1000 mL with ddH$_2$O.

The solution can be stored at 4°C for at least 3 months.

50- or 100-mL aliquots can be stored at −30°C for a long time.

Alternatives: 4% paraformaldehyde solution without picric acid can be used as the fixative solution. To avoid oxidation of the paraformaldehyde without picric acid, we recommend the solution to be used within a week (storing at 4°C until use). The solution can be stored at −30°C for a long time.

⚠ CRITICAL: Preparation of the fixative solution should be performed under the fume hood. To avoid scattering of paraformaldehyde powder, we recommend to use a weighing dish rather than a weighing paper for measuring the powder. Picric acid is not only toxic but also explosive when dried. Do not keep the lid open especially under the fume hood.

3% agar solution (for embedding a fixed brain)

Reagent	Final concentration	Amount
Agar	3% (w/v)	6 g
Agarose S	0.3% (w/v)	0.6 g
0.1 M PB	N/A	Fill up to 200 mL
Total	N/A	200 mL

Dissolve agar and agarose S by boiling the solution using microwave radiation.
Aliquot the solution into 50-mL conical tubes and keep until the liquid becomes solid.
Solid agar can be stored at 4°C for at least 3 months.

Citrate-based antigen retrieval solution (pH 6.0)

Reagent	Final concentration	Amount
Trisodium citrate	10 mM	2.94 g
ddH$_2$O	N/A	Fill up to 1,000 mL
Total	N/A	1,000 mL

Adjust pH to 6.0 by adding 1 M HCl (nearly 9 mL for the 1,000-mL solution).
The solution can be stored at 20°C–25°C for 1 year.

For antigen retrieval, boil the solution using microwave irradiation.

Soak sections in it and keep at 90°C for 10–30 min in the laboratory incubator.

After that, return it to 20°C–25°C and carry out performing immunohistochemistry.

Alternatives: As another option, the Tris-EDTA solution can be used for antigen retrieval. Add 1.21 g Tris base and 0.372 g EDTA in 1,000 mL ddH$_2$O and adjust pH to 9.0 by adding 1 M NaOH (nearly 0.3 mL). The solution can be stored at 20°C–25°C for 1 year.

10% normal donkey serum with 0.3% Triton X-100

Reagent	Final concentration	Amount
Normal donkey serum	10% (v/v)	1 mL
20% Triton X-100	0.3% (w/v)	150 μL
0.1 M PB	N/A	9 mL
Total	N/A	10 mL

The solution can be stored at 4°C for 1 month.

10% sucrose solution

Reagent	Final concentration	Amount
Sucrose	10% (w/v)	10 g
0.1 M PB	N/A	Fill up to 100 mL
Total	N/A	100 mL

The solution can be stored at 4°C for 1 month.

30% sucrose solution

Reagent	Final concentration	Amount
Sucrose	10% (w/v)	30 g
0.1 M PB	N/A	Fill up to 100 mL
Total	N/A	100 mL

The solution can be stored at 4°C for 1 month.

Antifreeze solution

Reagent	Final concentration	Amount
Glycerol	30% (w/v)	150 g
Ethylene glycol	30% (v/v)	150 mL
NaCl	0.85% (w/v)	4.25 g
0.4 M TB*	0.04	50 mL
ddH$_2$O	N/A	Fill up to 500 mL
Total	N/A	500 mL

The solution can be stored at −30°C for a long time.
*For preparation of 0.4 M TB, dissolve 4.85 g Tris-HCl and 1.11 g Tris base in 100-mL ddH$_2$O.

Gelatin solution for preparing gelatin-coated glass slides

Reagent	Final concentration	Amount
Gelatin	1% (w/v)	2 g
Chromium (III) Potassium Sulfate·12H$_2$O	0.005% (w/v)	10 μg
ddH$_2$O	N/A	200 mL
Total	N/A	200 mL

Add chromium (III) potassium sulfate after melting gelatin by heating the solution at 60°C and filter the solution. We recommend to prepare the solution on the day of use.
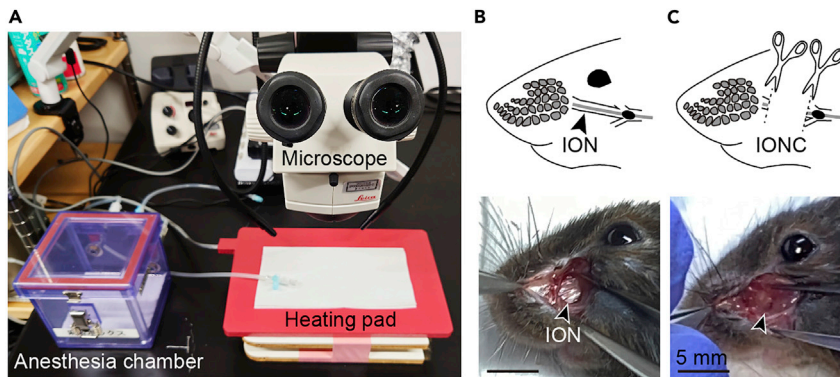
Figure 1. Development of mouse model for the infraorbital nerve (ION) cut
(A) A setup for mouse surgery.
(B) Exposed ION. Scale bar, 5 mm.
(C) No running ION after ION cut (IONC). Scale bar, 5 mm.

After washing glass microscope slides with 70% ethanol and then with ddH2O, dip them into the warm gelatin solution keeping at 60°C.

Dry gelatin-coated glass slides in the laboratory incubator at 40°C for 12–16 h (at least for 4–6 h).

STEP-BY-STEP METHOD DETAILS
Development of mouse model for the infraorbital nerve injury

⏱ Timing: 15–20 min

In the mouse whisker somatosensory pathway, thalamic neurons undergo synaptic remodeling not only during development but also after peripheral nerve injury. To examine structural and functional changes in synapses after peripheral nerve injury, we perform transection of the infraorbital nerve, which is the second branch of the trigeminal nerve and conveys tactile information from the whiskers.

1. Mouse anesthesia
   a. Anesthetize a mouse (older than 3 weeks) by 2–3% isoflurane in an anesthesia induction chamber for 5 min (Figure 1A).
   b. Anesthesia is maintained by intraperitoneal injection of a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg) in saline (0.9% NaCl).

   Note: We use C57 BL/6 wild type mice or transgenic mice produced from C57 BL/6 genetic background of both sexes for this study. Synaptic remodeling in the thalamus is observed both in male and female mice. Mice in the age range of 3–8 weeks old, which corresponds to the period after the developmental synapse remodeling and maturation in the whisker thalamus (Takeuchi et al., 2014), are used.

2. Unilateral digestion of the infraorbital nerve
   a. Place a mouse on a heating pad (40°C) (Figure 1A) and make a vertical incision between the whisker pad and the eye (Figure 1B).
   b. Expose the infraorbital nerve under a microscope using two forceps (Figure 1B). The nerve should be freed from the connective tissue without bleeding. Troubleshooting 1
   c. Locally anesthetize the nerve by applying 2% xylocaine with 1:80,000 adrenaline on the surface of the nerve using a 1-mL syringe with a 26- or 27-gauge needle.
   d. Lift the nerve up and cut the nerve at a central site first and then a peripheral site at a distance of 1–2 mm from the central site to prevent axonal regeneration (Figure 1C).
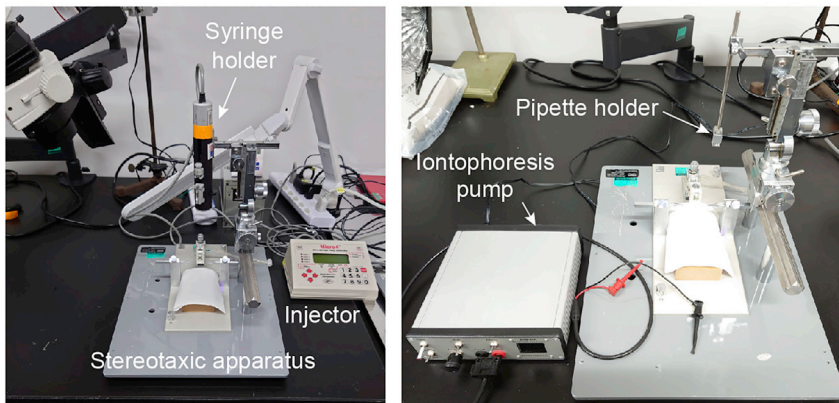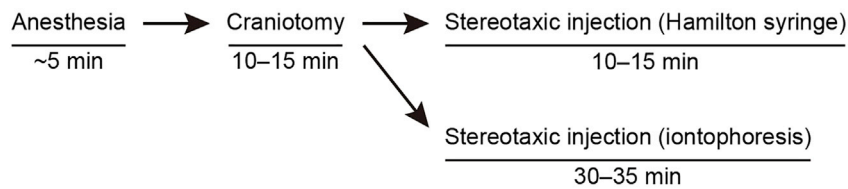
Figure 2. The timing and workflow of stereotaxic injection

3. Postoperative care
    a. After suturing the skin, inject atipamezole (0.1 mg/kg) intraperitoneally to reverse the sedative effect of medetomidine.
    b. Return the mouse to the home cage and house until the electrophysiological or histological experiment is performed.

Note: Synaptic remodeling in the thalamus is induced 5–6 days from the nerve cut (Takeuchi et al., 2012).

⚠ CRITICAL: Operation should be terminated within 30 min because sedative effect of medetomidine usually continues only 30 min from the intraperitoneal injection. We recommend continuous administration of 0.5–1% isoflurane (or sevoflurane) in addition to medetomidine injection.

Stereotaxic injection

⊙ Timing: 25–60 min

Refer to ''materials and equipment'' for information about reagents used. The timing and workflow are depicted in Figure 2.

4. Mouse anesthesia
    a. Anesthetize a mouse by 2–3% isoflurane in an anesthesia induction chamber for 5–10 min (Figure 1A).
    b. Anesthesia is maintained by intraperitoneal injection of a mixture of medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg) in saline (0.9% NaCl).

Note: When the operation is expected to be prolonged (more than 25 min), apply 0.5–1% isoflurane continuously to maintain anesthesia. Body temperature should be monitored and maintained during the operation.

5. Craniotomy
   a. Place the anesthetized mouse in a stereotaxic apparatus using an auxiliary ear bar (Figure 2A). Apply 2% xylocaine jelly to the outer ear for local anesthesia using a swab before the auxiliary ear bar is attached.
   b. Apply 0.3% ofloxacin gel to both eyes using a swab to protect eyes during the operation.
   c. Make a skin incision to expose a skull after local anesthesia with 2% xylocaine jelly application.
   d. Adjust the nose position using an incisor bar until z coordinates of bregma and lambda become the same.
   e. Drill the skull above the marked sited using a micromotor system with a steel bur. To reduce heat damage of the brain, the drilling should be paused by cooling with saline of 20°C–25°C. Troubleshooting 2

   Note: Diameter of the drilled hole is typically around 0.5 mm.

6. Injection using a Hamilton syringe (Figure 2)
   a. Attach a 10 μL Hamilton syringe with a stainless-steel needle to a syringe holder connected with a microprocessor-controlled injector.

   △ CRITICAL: We recommend the use of a 35-gauge beveled needle to reduce brain damage. After the injection, the Hamilton syringe with a needle should be washed 2–3 times with water and then with 70% ethanol.

   b. Fill the injection solution into the needle from the tip.
   c. Move the syringe to the surface of the drilled site.
   d. Insert the needle into the target depth of the brain and inject the solution continuously.
   e. Remove the needle.

   Note: After the injection, we recommend the needle remained in place for at least 5 min before being withdrawn. Volume of the injection solution typically ranges between 100 and 500 nL. Injection is completed within 5 min. The speed of the injection is adjusted to 40–100 nL/min.

   f. Fill the drilled hole with hemostatic gelatin sponge (e.g., Spongel, see ''key resources table'') and close the scalp wound.
   g. Inject atipamezole (0.1 mg/kg) intraperitoneally to reverse the sedative effect of medetomidine.
   h. Return the mouse to the home cage and house until the electrophysiological or histological experiment is performed.

   Note: Injection by air pressure can be performed using Picospritzer as described in the recent protocol (Okamoto et al., 2020). When you need to inject into more restricted site or you need to reduce neuronal damages, iontophoretic delivery is available as described below (step 7).

7. Iontophoretic delivery using a glass pipette (Figure 2)
   a. Pull borosilicate glass capillaries (1 or 1.5 mm O.D.) using a single pull with a micropipette puller (P1000 or P97, Sutter Instrument).

   Note: We use a single cycle for pulling an injection pipette using the micropipette puller (P-1000 or P-97, Sutter Instrument) to obtain pipettes with long tapers (e.g., step 1: heat, ramp; pull, 50; velocity, 200; delay, 250; pressure, 500).

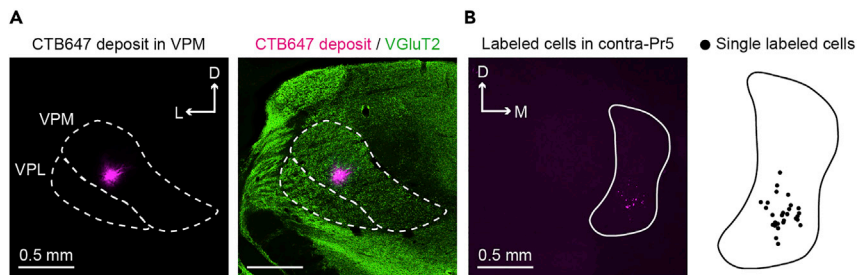   b. Cut off the tip of a pulled-pipette using scissors (tip diameter, 20–50 μm).

Figure 3. Focal injection using iontophoresis

(A) A retrograde tracer, Alexa Fluor 647-conjugated cholera toxin subunit B (CTB647, 0.2% in ddH$_2$O), is iontophoretically delivered to the dorsal part of the thalamic ventral posteromedial nucleus (VPM). VPM is identified by VGLUT2 immunohistochemistry using a guinea pig anti-VGluT2 antibody and an Alexa Fluor 488-conjugated secondary antibody. Gray-scale images are acquired using an epifluorescence microscope (Axio Scope.A1, Carl Zeiss) equipped with a cooled CCD camera (QSI RS 6.1, Quantum Scientific Imaging) and pseudocolored using Adobe Photoshop software. D, dorsal; L, lateral; VPL, ventral posterolateral nucleus. Scale bar, 0.5 mm.

(B) Retrogradely labeled cells in the ventral part of the principal trigeminal nucleus of the brainstem (Pr5) in the contralateral hemisphere. M, medial. Scale bar, 0.5 mm.

    c. Draw the injection solution into the pipette tip by capillary action and backfill saline slowly using the 1-mL syringe with a flexible needle. Troubleshooting 3

    d. Insert an Ag/AgCl wire into the pipette and attach the pipette to the pipette holder.

    e. Move the pipette to the surface of the drilled site.

    f. Insert the pipette into the target depth of the brain and apply current with 7-s on/off cycles for 20–30 min using an iontophoresis pump. Troubleshooting 4

   ⚠ CRITICAL: Current polarity (positive or negative) depends on the reagent and the solvent used (e.g. ddH$_2$O, saline, or 0.1 M PBS). Current intensity depends on the tip diameter or the target region and usually ranges between 0.3–5 μA. To make focal injection, lower current intensity is suitable for injection into the neocortex (Ueta et al., 2014), while larger one is necessary for injection into dense structures such as the thalamus and brainstem (Ueta and Miyata, 2021).

    g. Remove the pipette. Leaving the pipette before withdrawn is not necessary when the diameter of the pipette is 20 μm, because the injection solution does not leak out from such narrow tip (Figure 3).

    h. Carry out postoperative care as described above.

   Note: For local depletion of microglia, PLX3397, which inhibits microglial proliferation, or clodronate liposomes, which induces microglial apoptosis, is available (Figure 4). For local activation of microglia, lipopolysaccharides is available (refer to ''key resources table'') (Ueta and Miyata, 2021). Genetic regulation of local microglia by such as the diphtheria toxin receptor expression system using transgenic mouse lines (Willis and Vukovic, 2020), or virally-introduced DREADD system are also useful (Grace et al., 2018). These transgenic mouse lines or DREADD expressing viral vectors under microglial promoter are commercially available from the Jackson Laboratory or Addgene.

Focal injection using iontophoresis is also useful for neuronal tracing from a restricted area (Ueta et al., 2014). For example, we can successfully obtain retrogradely labeled cells in the whisker region in the brainstem, which corresponds to the ventral part of the principal trigeminal nucleus (Pr5), by a small injection (100–200 μm of the diameter) restricted in the whisker thalamus, which corresponds to the dorsal part of the ventral posteromedial nucleus (VPM) (Figure 3). In this protocol, Alexa Fluor 647-conjugated cholera toxin subunit B is used as a retrograde tracer (0.2%, dissolved in 0.1 or 0.05 M PBS) (Figure 3). We
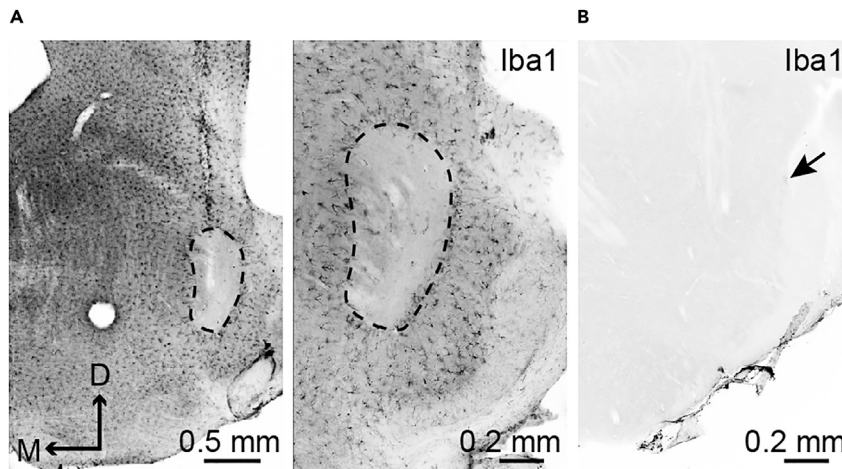
Figure 4. Local and brain-wide depletion of microglia

(A) Clodronate liposomes, which induce apoptosis of microglia, are locally injected to the brainstem using a Hamilton syringe. No immunoreactivity against a myeloid cell marker Iba1 is found in the injection site. Immunohistochemistry is performed using a rabbit anti-Iba1 antibody and an Alexa Fluor 488-conjugated secondary antibody. Gray-scale images are acquired using the epifluorescence microscope same as in Figure 3 and inverted using Adobe Photoshop software. Scale bar, 0.5 mm (left) or 0.2 mm (right).

(B) Brain-wide microglia are depleted by systemic administration of PLX3397, which inhibits microglial proliferation. An arrow indicates a remaining Iba1-positive cell in the brainstem. Scale bar, 0.2 mm.

have previously utilized Alexa Fluor 555-conjugated cholera toxin subunit B (0.2%) and obtained successful retrograde labeling from the restricted cortical layers (Ueta et al., 2014).

Whole-cell patch-clamp recording using acute brain slices

⏱ Timing: ≥2 h

The timing and workflow are depicted in Figure 5.

8. Preparation of solutions (refer to ''materials and equipment'')
   a. 10× aCSF stock solution
   b. 1M $CaCl_2$ solution
   c. 1M $MgCl_2$ solution
   d. Cutting solution
   e. Intracellular pipette solution for voltage-clamp or current-clamp recording

9. Preparation of acute brain slices
   a. Dilute the 10× aCSF stock solution to 1 L with ddH_2O, add 3.6 g glucose (20 mM), and bubble with a mixture of 95% O_2 and 5% CO_2.
   b. Add 2 mL of 1M $CaCl_2$ solution (2 mM) and 1 mL of 1M $MgCl_2$ solution (1 mM).
   c. Transfer 100–150 mL of aCSF to a glass beaker and place it in the water bath adjusted to 32°C. Bubble this aCSF solution continuously.
   d. Bubble the cutting solution on ice.

   Note: To improve recovery of neurons, add Na-ascorbate (5 mM), Na-pyruvate (3 mM), and thiourea (2 mM) to both aCSF and the cutting solution. All of Na-ascorbate, Na-pyruvate, and thiourea should be removed from aCSF during recording. Alternatively, add lactic acid (4 mM) instead of Na-ascorbate, Na-pyruvate, and thiourea (Ueta et al., 2019). Lactic acid is also removed from aCSF during recording.
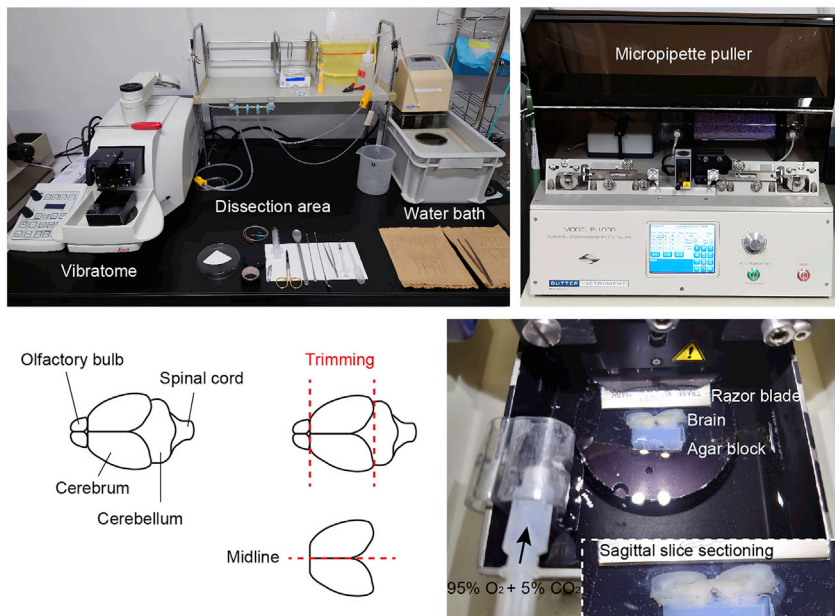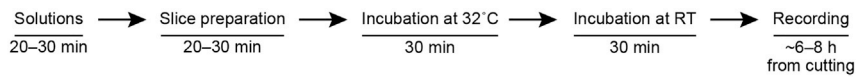
Figure 5. The timing and workflow of preparation of acute brain slices

e. After a deep anesthesia of a mouse with isoflurane, perfuse 5 mL of the cooled cutting solution transcardially using a 10 mL syringe with a 21-gause syringe needle within 1 min.

Note: More details about transcardial perfusion are described in the step 13.

f. Decapitate, remove the brain, and soak it into the cutting solution on ice for 1–3 min.

⚠ CRITICAL: Removing the brain and soaking it in the air-bubbled cutting solution after decapitation should be done quickly to avoid neuronal damages or death.

g. Trim the brain on a filter paper using a razor blade or a surgical blade.
h. Glue the brain block onto the electrical-taped specimen disk using cyanoacrylate (Aron alpha). Glue a block of gelatin (2–3%) behind the brain block. Place the disk onto the buffer tray with the cutting solution continuously bubbled with 95% O_2 and 5% CO_2.
i. Cut sagittal slices of 250- or 300-μm thickness including the whisker thalamus (VPM) using a vibratome with a razor blade and collect on the holding chamber in the water bath. Cutting speed is basically 0.04 mm/s (VT1200S, Leica Microsystems). Frequency amplitude normally ranges between 0.4 and 0.5 mm (VT1200S). Troubleshooting 5

Note: A double edge razor blade is cut in half using tinner's snips and is attached to blade holder of the vibratome. We can cool the buffer tray with ice or icepacks and apply the sherbet-like chilled cutting solution in the buffer tray. Temperature during slice cutting may affect various physiological properties probably depending on the cell type or the age of animals (e.g., Eguchi et al., 2020). It is important to select appropriate methods depending on the application or the purpose of experiments.
j. After cutting, incubate brain slices in the aCSF solution for 30 min at 32°C using a water bath and then for at least 30 min at 20°C–25°C until use.
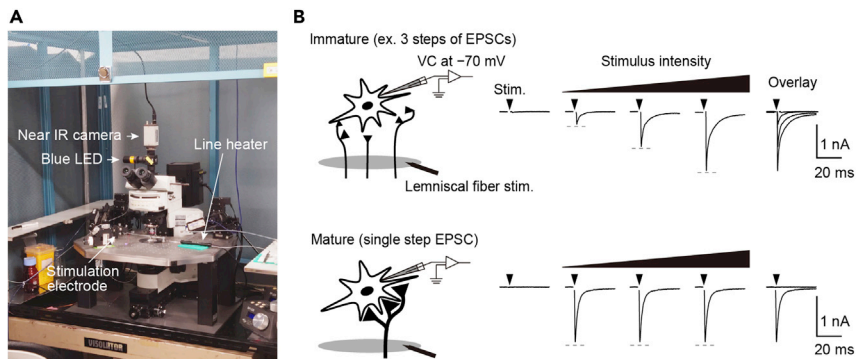
Figure 6. A setup for whole-cell patch clamp recording and example experiments
(A) A setup for patch-clamp experiments.
(B) Estimation of innervated axon numbers by excitatory postsynaptic current (EPSC) recording from a thalamic VPM neuron under voltage-clamp.

> *Note:* Electrophysiological recording is normally finished within 6–8 h from the slice cutting. To obtain synaptic responses, we recommend to finish recordings within 4–6 h from the decapitation.

10. Assessment of number of innervated axons by recording of excitatory postsynaptic currents (EPSCs) in individual neurons of the whisker thalamus (Figure 6)
    a. To block inhibitory postsynaptic currents, add $GABA_A$ receptor antagonist bicuculline methochloride (10 μM) and $GABA_B$ receptor antagonist CGP55845 hydrochloride (1 μM) to the perfusing aCSF continuously bubbled with 95% O_2 and 5% CO_2. Temperature of aCSF is maintained at 31°C–32°C using a line heater (Figure 6A).
    b. Establish a whole-cell patch on a thalamic neuron which is identified using a near-infrared camera (C3077-79, Hamamatsu Photonics)-equipped microscope (BX51WI, Olympus Corp.) with a 60× water-immersion objective (numerical aperture = 0.9, Olympus Corp.).

> *Note:* VPM is visually identifiable in the bright-field images (Takeuchi et al., 2012; 2014). VPM in mice consists of glutamatergic thalamocortical neurons and is mostly devoid of local interneurons.

    c. EPSC is evoked by an electrical stimulation using a concentric electrode placed on the lemniscal fiber bundle.
    d. The number of EPSC steps corresponds to the number of axons innervating a recorded neuron. For example, a single neuron which receives three axonal inputs expresses three EPSC steps when a stimulus intensity is gradually increased, but a neuron receiving a single axonal input expresses a single EPSC step in an all-or-none fashion (Figure 6B).

> *Note:* We use 5 or 6 cycles for pulling a whole-cell patch pipette (4–8 MΩ) using the micropipette puller to obtain pipettes with short tapers (e.g., step 1: heat, ramp; pull, 0; velocity, 19; delay, 1; pressure, 500; step 2: ramp – 5; pull, 0; velocity, 19; delay, 1; steps 3 and 4: heat, ramp – 15; pull, 0; velocity, 19; delay, 1; steps 5 and 6: ramp – 25; pull, 0; velocity, 20; delay, 1). We usually increase or decrease velocity in the last step to correct the tip diameter. The internal solution is kept on ice during recording. A recent detailed protocol for patch-clamp techniques would help your experiments (Manz et al., 2021).

> ⚠ CRITICAL: Neurons in the mouse whisker thalamus receive glutamatergic excitatory inputs from lemniscal fibers, which originate from the brainstem, and/or from corticothalamic axons. EPSCs evoked by these two different pathways have different EPSC kinetics and short-term plasticity: lemniscal fiber-evoked EPSCs, fast EPSC decay and paired-pulse
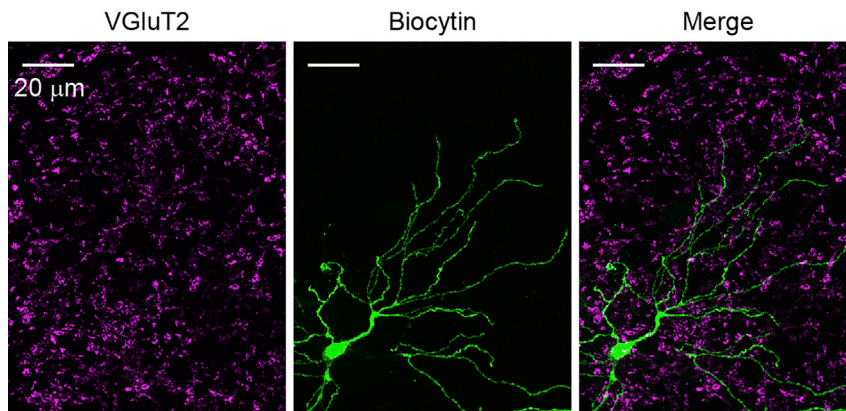
Figure 7. Post hoc visualization of cell morphology after electrophysiological recording
A VPM neuron is visualized with Alexa Fluor 488-conjugated streptavidin. Immunohistochemistry against vesicular glutamate transporter type 2 (VGluT2) and Alexa Fluor 647-conjugated secondary antibody is simultaneously performed. Gray-scale images are acquired using a fluorescent microscope (BZ-X810, Keyence) and pseudocolored. The slice is resectioned at 40 μm. Scale bar, 20 μm.

depression; corticothalamic EPSCs, slower decay of EPSCs and paired-pulse facilitation (Miyata and Imoto, 2006).

11. Post hoc visualization of dendritic and axonal morphologies of a recorded neuron.
    a. After recording, the slices of 250- or 300-μm thickness are collected in a glass vial or a 24-well plate, soaked into the fixative solution, and stored for 12–16 h at 4°C. The fixative solution of 2 mL is enough for a single slice.

Note: The slices can be stored in the fixative solution at 4°C for few weeks. It can be possible to store for longer time (e.g., 2–3 months).

    b. Wash the slices three times with 0.1 M PBS for 10–20 min on a shaker.
    c. Incubate the slices with fluorescent streptavidin (e.g., Alexa Fluor 488-conjugated streptavidin, 1:500) in 0.1 M PBS with 0.05% Triton X-100 for 2–3 h at 20°C–25°C or 12–16 h at 4°C.
    d. Wash the slices two times with 0.1 M PBS for 5–10 min and then with 0.1 M PB for 5–10 min to remove NaCl.
    e. Mount the slices on a gelatin-coated glass slide (refer to ''materials and equipment'').

Note: Use spacers (two sheets of electrical tape, 200 μm thickness for one sheet) when sections are coverslipped not to flatten the slices.

    f. Seal the sections with antifade mountant and apply a coverslip.

Note: After fixation, the sections can be resectioned at a thickness of 40 or 50 μm. Such thinner sections are useful for simultaneous immunohistochemical staining (Nagumo et al., 2020) (Figure 7).

Immunohistochemistry of fixed brain tissue

⊙ Timing: 2–3 days

To identify whisker-originated axon terminals in the whisker thalamus, we have crossed a Krox20-Cre mouse and an Ai34D reporter mouse, which expresses synaptophysin-tdTomato under Cre inducing conditions (Figure 8). In the brainstem trigeminal nucleus of Krox20-Cre mice, Cre is expressed in
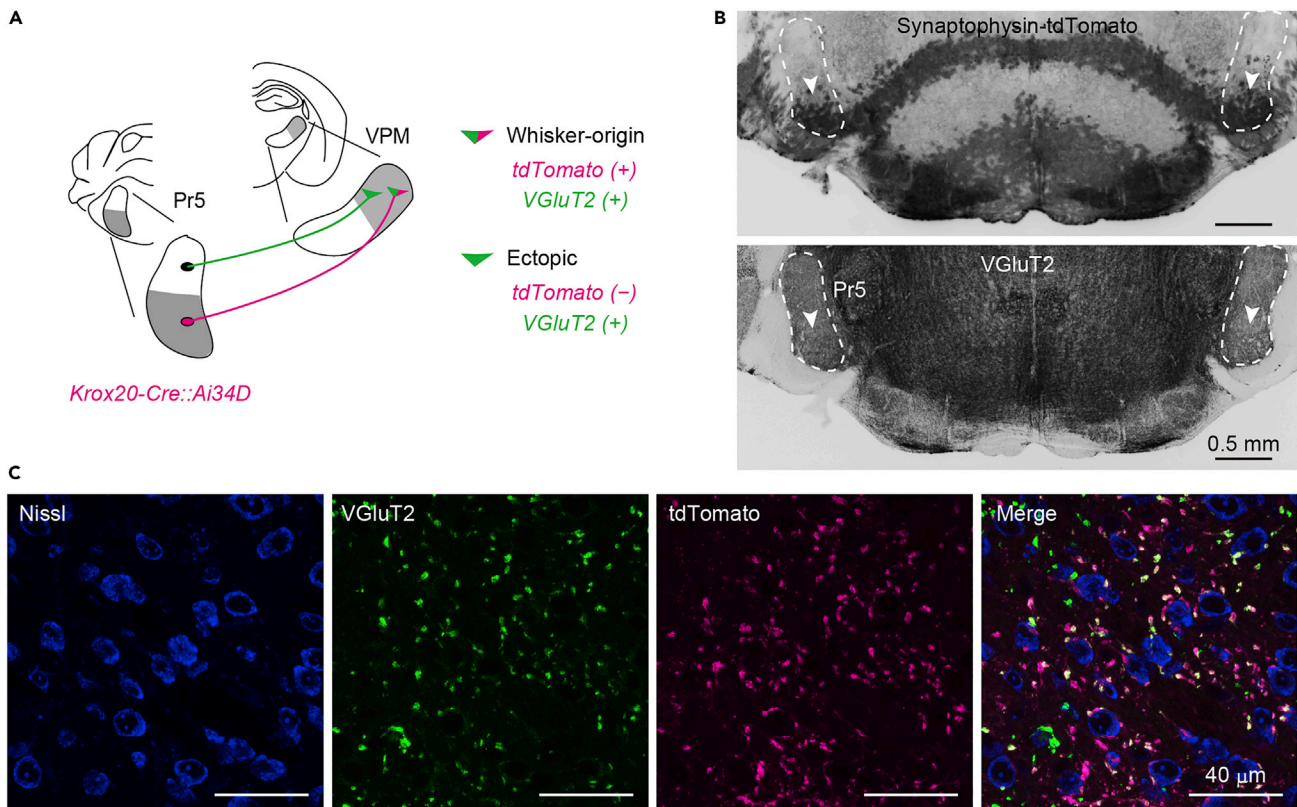
Figure 8. Schematic illustrating the identification of pathway-specific axon terminals in the thalamus and example sections

(A) Identification of whisker-origin and ectopic (non-whisker-origin) axon terminals in the thalamic VPM using Krox20-Cre mice crossed with Ai34D mice, which express synaptophysin-tdTomato under Cre.

(B) Cre recombinase is expressed in principal neurons in the ventral whisker region of Pr5. Scale bar, 0.5 mm.

(C) Axon terminals in VPM. Gray-scale images are acquired using a confocal laser scanning microscope (LSM710, Carl Zeiss) and pseudocolored. Scale bar, 40 μm.

thalamus-projecting principal neurons in the whisker region but not in neurons in non-whisker regions (Bechara et al., 2015; Takeuchi et al., 2014; Voiculescu et al., 2000).

The timing and workflow are depicted in Figure 9.

12. Preparation of solutions (refer to ''materials and equipment'')
    a. 0.1 M PB and PBS
    b. 0.05 M Tris buffer (TB) and Tris-buffered saline (TBS)
    c. Prefixative solution
    d. Paraformaldehyde-based fixative solution
    e. Agar solution for embedding a brain block
    f. Antigen retrieval solutions
    g. 10% normal donkey serum solution with 0.3% Triton X-100
    h. 10% or 30% Sucrose solution
    i. Antifreeze solution

13. Preparation of fixed brain tissue
    a. Deeply anesthetize the *Krox20-Cre : Ai34D* mouse with isoflurane and place it on the shallow tray.
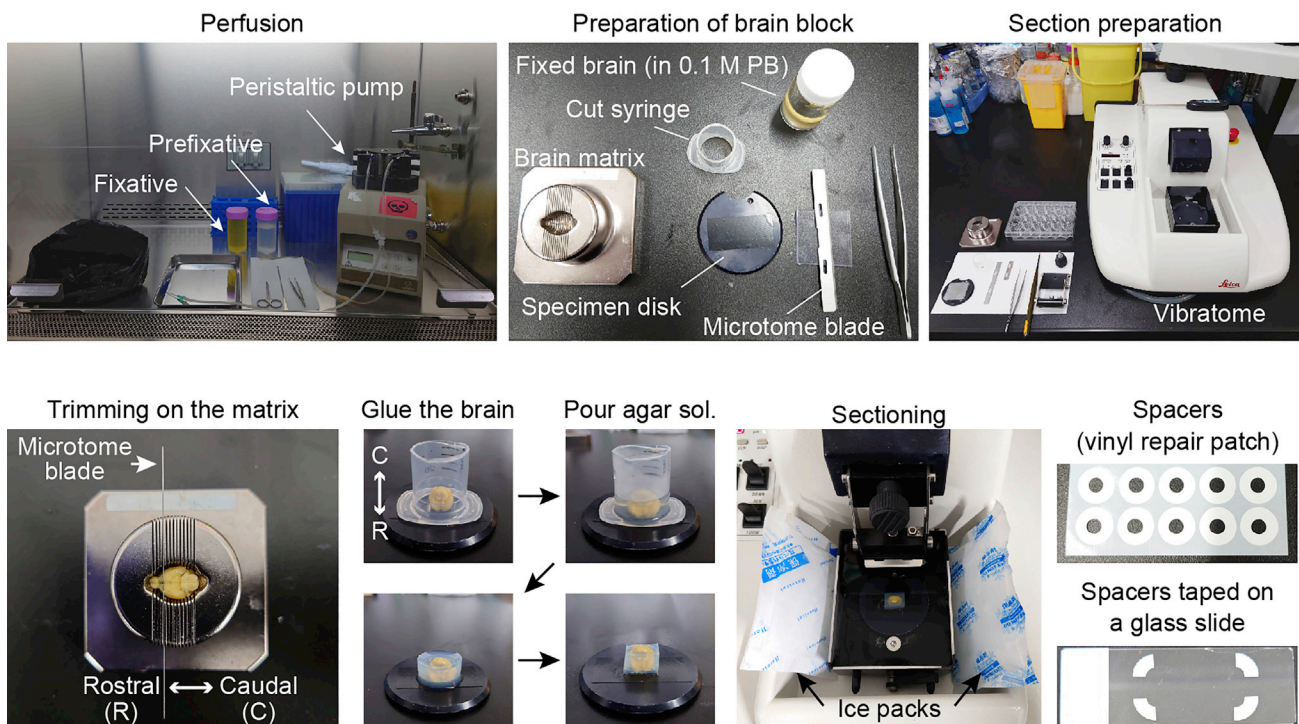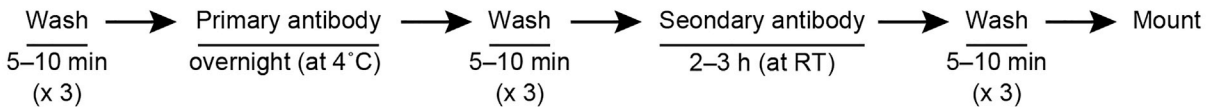   b. Make an abdominal incision on the liver and find the xiphoid process.

<Animal fixation and section preparation>

Anesthesia $\longrightarrow$ Perfusion $\longrightarrow$ Postfixation at 4°C $\longrightarrow$ Section preparation $\longrightarrow$ Storage

~5 min   15–20 min   30 min ~ overnight   30–60 min

<Immunohistochemistry (free-floating)>

Wash $\longrightarrow$ Primary antibody $\longrightarrow$ Wash $\longrightarrow$ Seondary antibody $\longrightarrow$ Wash $\longrightarrow$ Mount

5–10 min   overnight (at 4°C)   5–10 min   2–3 h (at RT)   5–10 min

(x 3)   (x 3)   (x 3)

Figure 9. The timing and workflow of immunohistochemistry using fixed brain sections

c. Lift up the xiphoid process, expose the pleural cavity by making a lateral incision in the diaphragm, and cut along the lateral wall of the rib cage on both sides until both the heart and the thymus, which is in front of and above the heart, are exposed.

Note: To avoid injuring the lung, carefully displace it during cutting the rib cage.

d. Make a small incision in the right atrial ear and quickly insert a 21-gauge needle, which connects to the peristaltic pump system (Figure 9), to the left ventricle of the heart.

Note: You can insert the needle to the left ventricle first and cut the right atrial ear next.

e. Perfuse the fixative solution followed by the 5 mL of the prefixative solution at 20°C–25°C for 10–15 min using a peristaltic pump. Troubleshooting 6

Note: The volume of the fixative solution is twice the body weight.

f. Brains are usually post-fixed in the fresh fixative for 30 min or 12–16 h at 4°C. After post-fix-ation, the fixative solution is replaced by 0.1 M PB.

▯▯ **Pause point:** Although the fixed brain can be stored in 0.1 M PB for few weeks at 4°C, we recommend to prepare thin sections within few days from the perfusion.

14. Preparation of thin sections using a vibratome
    a. Trim the fixed brain on the brain slicer matrix using a microtome blade.
    b. Glue the brain block onto the specimen disk using Aron Alpha. Mount the block with agarose gel solution using a mold (20 mL cut syringe). Allow the disk to cool to set the gel in the refrig-erator.
    c. Remove the mounted brain block from the disk after trimming gels and glue onto the spec-imen disk again.

Note: By this step, the brain block embedded in agarose gel is tightly glued onto the disk.

    d. Set the disk onto the buffer tray with cooled 0.1 M PB.
    e. Cool the buffer tray with ice or ice packs during cutting using a vibratome with an injector blade. Cutting speed ranges between 0.05–0.2 mm/s (scale 1–4.5 for Leica VT1000S). Fre-quency is normally set at 40–50 Hz (scale 4–5).
    f. Collect sections and submerge them into a 24-well plate with 0.1 M PB.

Note: To observe both the brainstem and the thalamus, we usually use coronal sections for histological experiments. Section thickness is usually set to 40 or 50 μm for handling and anti-body penetration. We use sections of 8–20 μm thickness for analyzing colocalization of different markers or labeling in individual cell somata using single-focus images under epi-fluorescence microscopy.

▯▯ **Pause point:** Sections may be stored at 4°C for a couple of weeks, but we recommend that sections are immersed in an antifreeze solution after a sucrose-substitution procedure and kept at −30°C for long-term storage. Use a cryostat to obtain thinner sections after a su-crose-substitution procedure (10% sucrose for 4–6 hours to 12–16 h and 30% sucrose for one day or few days at 4°C).

15. Fluorescent immunohistochemistry on free-floating sections
    a. Wash the sections three times with 0.1 M PBS for 5–10 min in a 12-well plate on a shaker.
    b. Incubate the sections with primary antibody (guinea pig polyclonal antibody against vesicular glutamate transporter type2 (VGluT2), 1:500 or 1,000) in 10% normal donkey serum solution with 0.3% Triton X-100 for 12–16 h at 4°C or 2–3 h at 20°C–25°C on an orbital shaker.

Note: Use 0.4–0.5 mL of the antibody solutions for a single well in a 12-well plate (less than 6 sections per well). Incubation with primary antibodies is usually carried out for 12–16 h at 4°C.

Alternatives: For VGluT2 immunohistochemistry, rabbit polyclonal antibody is also available and working well (refer to "key resources table").

    c. Wash the sections three times with 0.1 M PBS for 5–10 min.
    d. Incubate the sections with secondary antibody (Alexa Fluor 488-conjugated antibody against guinea pig IgG, 1:500) and Fluorescent Nissl (NeuroTrace 435/455, 1:100) in 10% normal donkey serum solution with 0.3% Triton X-100 for 12–16 h at 4°C or 2–3 h at 20°C–25°C on an orbital shaker.

Note: Incubation with secondary antibodies is usually carried out in a dark place for 2–3 h at 20°C–25°C.

Alternatives: NeuN immunohistochemistry is also useful for visualizing neurons (refer to "key resources table").

   e. Wash the sections two times with 0.1 M PBS for 5–10 min and then with 0.1 M PB for 5–10 min.
   f. Mount the sections on a gelatin-coated glass slide.
   g. Seal the sections with antifade mountant and apply a coverslip. Troubleshooting 7
   h. Acquire images using an epifluorescent microscope or by confocal microscopy. Troubleshooting 8

Note: Use spacers (Vinyl repair patch, 70 μm thickness) when sections are coverslipped for confocal microscopic observation.

When we need to visualize myeloid cells including microglia, we use anti-Iba1 antibody for immunohistochemistry (refer to "key resources table") (Figure 4). Because anti-Iba1 antibody detects diverse type of myeloid cells, we recommend to use anti-TMEM119 antibody for detecting microglia specifically (refer to "key resources table") (Ueta and Miyata, 2021).

Alternatives: When we only need to acquire lower magnification images (e.g., 2×, 4×, or 5×), we use an aqueous mountant without antifade reagents (e.g., Fluoromount-G).

EXPECTED OUTCOMES

Infraorbital nerve cut (IONC) induces microglial accumulation in the whisker region of the brainstem but not in the thalamus (Ueta and Miyata, 2021). Using stereotaxic injections, we can manipulate local microglial activity as well as neuronal activity (for genetic approaches, refer Willis and Vukovic, 2020). Systemic microglial depletion using oral administration of microglial inhibitor is also commercially available and successful (Elmore et al., 2014; Ueta and Miyata, 2021). Combined these local and systemic regulation of microglia, the understanding of the role of microglia in various developmental and pathophysiological conditions will be progressed.

To understand the mechanisms for synaptic remodeling, it is important to know both structural and physiological characteristics of synapses. A whole-cell patch-clamp recording from an identified single neuron in acute brain slices has some limitations described below, but is still useful for detailed analyses of synaptic properties including kinetics and structure; for example, we can estimate the number of innervated axons by counting EPSC steps. In addition, dendritic and axonal morphologies can be reconstructed by biocytin or neurobiotin filling during recording and post hoc visualization (Ueta et al., 2019; Ueta and Miyata, 2021). Combined electrophysiological and anatomical techniques, together with utilization of region-specific Cre mice, are advantageous strategy to label and manipulate specific circuits or projection pathways.

LIMITATIONS

Depletion or activation effect of microglia by using a single focal injection will not continue for a long-time period. We have observed that microglia are still depleted at the injection site after 5–6 days from a single injection of clodronate liposomes (Ueta and Miyata, 2021). Genetic approaches using microglia-targeted recombinant viral infection probably overcome this limitation. In addition, genetic approaches enable targeting different microglial population with activation of state-specific gene expression (Friedman et al., 2018; Hammond et al., 2019). Repopulation of microglia is another important issue when microglia are systemically or locally depleted, because repopulated microglia have both neurotoxic and neuroprotective effects (Rubino et al., 2018; Willis et al., 2020), and also

because the rate of microglial repopulation depends on the extent of depletion (Najafi et al., 2018). Long-term effects of microglial manipulation will be highlighted in future studies.

It is difficult to know whether individual axonal input originates from the whisker region or non-whisker regions in electrophysiological recording. However, using Cre-off viral injection into Krox20-Cre mice combined with direct recording from an identified presynaptic terminal, we have recently enabled the pathway-specific measurement of electrophysiological properties in acute slices (Midorikawa and Miyata, 2021).

TROUBLESHOOTING
Problem 1
Bleeding occurs intraoperatively (step 2).

Potential solution
Add one drop of saline or 2% xylocaine with 1:80,000 adrenaline using a 1-mL syringe with a 26- or 27-gauge needle to stop bleeding.

Problem 2
Bleeding occurs during drilling (step 5).

Potential solution
Place an absorbent cotton piece wet with warmed saline for a few minutes to stop bleeding. Rapid vibrations of the hand motor will cause bleeding from the skull. To minimize such vibrations, the hand motor can be controlled by a micromanipulator with a drill holder (e.g., Cat#SD-102, Narishige).

Problem 3
No capillary action (step 7).

Potential solution
The tip diameter is too narrow (probably smaller than 20 μm). Cut off the tip using scissors or gently break the tip using forceps.

Problem 4
No current flows (step 7).

Potential solution
It is probably due to an air bubble in the pipette. Tap the pipette gently not to mix the injection solution and backfilled saline to avoid reducing concentration of the injection solution. In our experiments, injection solution is filled within the tapering part of the glass capillary. Normally, an Ag/AgCl wire is incapable of inserting into the thin tapering part. So, backfilled saline is needed as the electric carrier. Alternatively, the injection solution can be backfilled. In that case, collect the solution after finishing the injection and use it in the next experiments.

Problem 5
Vibratome fails to accurately and repeatedly slice sections (step 9).

Potential solution
The brain block is not tightly glued onto specimen disk. We recommend to cut at low cutting speed (around 0.05 mm/s) after the brain block is tightly glued on the disk again. If that doesn't work, prepare thicker sections (e.g., 100 μm).

Problem 6
Uneven whole-brain fixation due to insufficient perfusion (step 13).

Potential solution
When the solution is released from the nose and/or the mouth during perfusion, it does not flow to the brain. This is probably due to clogged or ruptured blood vessels. To avoid this, remove air bubbles from a silicone tube connecting to a needle before inserting it into the left ventricle. We recommend to use a blunt-end needle, which can be made from a syringe needle by using diagonal and needle-nose pliers for example.

Problem 7
Undesirable air bubbles are formed under the coverslip (step 15).

Potential solution
After drying sections, place a generous amount (0.2–0.3 mL mountant for one slide) of mountant on the one side of the glass slide. The use of insufficiently small amount of mountant will result in air bubble formation. Hold one edge of a coverslip with fine tweezers (curved one is better) and place the other edge of the coverslip on the droplet. Slowly lower the tweezers together with the coverslip until the tip of the tweezers is close to the surface of the glass slide. Then, slowly pull out the tweezers from under the coverslip. Absorb excess mountant using a twisted paper (e.g., KimWipes). Finally, seal the coverslip with nail polish.

Problem 8
The signal is weak or the background of an acquired image is high (step 15).

Potential solution
There may be several causes. We recommend to perform antigen retrieval before antibody penetration or use TBS as a buffer instead of PBS (refer to ''materials and equipment''), because PBS is known to inhibit some enzymatic actions. Tyramine-based signal enhancement method will be helpful as described in a recent protocol (Okamoto et al., 2020).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoshifumi Ueta (yueta@twmu.ac.jp).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique data sets or code.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, writing – original draft, writing – review & editing, and funding acquisition, Y.U. and M.M.; investigation, Y.U.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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